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Carbonic Anhydrase III S-Glutathionylation Is Necessary for Anti-Oxidant Activity

P. Roy, M. Ireland, S. Roy, J. Craft, C. Bartholomew*

Department of Biological & Biomedical Sciences, School of Health & Life Sciences, Glasgow Caledonian University, City Campus, Glasgow, UK
Email: *c.bartholomew@gcu.ac.uk

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Abstract

Carbonic anhydrase isozyme CA3 protects cells against oxidative stress. Ectopic expression of murine Ca3, but not Ca2, protects proto-oncogene Evi1 expressing Rat1 fibroblast cells (ca3low) against hydrogen peroxide (H_2O_2) induced stress. Ca3 is S-glutathionylated via glutathione adducts with cysteines 181 and 186. Substitution of both Ca3 cysteines with serine fails to protect cells from oxidative stress. Insertion of cysteine at 181 and 186 in Ca2 is insufficient for conferring efficient anti-oxidant activity. This shows for the first time that S-glutathionylation of cys181 and cys186 residues is required for Ca3 anti-oxidant activity but that additional factors are also required.

Keywords

CA3, CAIII, Carbonic Anhydrase III, S-Glutathionylation, Apoptosis, Anti-Oxidant

1. Introduction

Intracellular pH is partly regulated by a family of carbonic anhydrase (CA, EC4.2.1.1) enzymes that reversibly catalyse the hydration of carbon dioxide to bicarbonate and hydrogen ions [1]. The *α*-CAs comprise 15 distinct zinc metalloenzymes that can be sub-divided into cytoplasmic (CAI, CAII, CAIII, CAVII, CAXIII), membrane bound (CAIV, CAIX, CAXII, CAXIV, CAXV), mitochondrial (CAV), non-catalytic (CAVIII, CAX, CAXI) and secreted (CAVI) [2]. These ubiquitous enzymes are of significant importance for many physiological processes and are implicated in various pathological conditions including atherosclerosis [3], retinitis pigmentosa [4], myasthenia gravis [5] and cancer [6].

Carbonic anhydrase III (CA3) is unique in this family as it has low hydratase activity [7] but it is very abundant in liver, skeletal muscle and adipose tissue.

However, surprisingly for such an abundant protein (e.g. 2% of wet weight in slow oxidative muscle (type 1) [8]) its function remains an enigma. Until recently Ca3KO mice were believed to lack any functional deficit [9] but have now been shown to display impaired mitochondrial ATP synthesis [10]. In addition, these mice show changes in expression of genes involved in oxidative stress [11]. This implies CA3 might participate in the cellular response to oxidative stress.

There is mounting evidence that strongly suggests CA3 is an anti-oxidant. Tissue in which it is abundant is those whose metabolic activity results in considerable oxidative stress, including aerobic respiration in skeletal muscle and lipid metabolism in adipose tissue. CA3 expression is co-induced with established anti-oxidant genes such as superoxide dismutase following endurance training in elite athletes when skeletal muscle is exposed to increased oxidative stress [12] [13].

Functional molecular evidence, in addition to the associated expression studies above, shows that CA3 has anti-oxidant activity. Enforced CA3 expression in NIH3T3 cells protects them from hydrogen peroxide (H_2O_2)-induced apoptosis [14]. EVI1 proto-oncoprotein transformed Rat1 fibroblasts have repressed ca3 expression and either transgene mediated restoration of Ca3 in these cells or direct RNAi mediated Ca3 KD in parental Rat1 cells (high endogenous ca3) protects and sensitizes cells to H_2O_2 induced apoptosis respectively [15]. Insight into a possible mechanism of CA3 anti-oxidant activity has been obtained from post-translational modification observed in cells during oxidative stress. CA3 is modified by S-glutathionylation in response to t-butylhydroperoxide or menadione in cultured hepatocytes [16] as well as in stressed skeletal muscle [11]. Crystal structure studies and site directed mutagenesis reveal two cysteine residues, cys181 and cys186, are available for the addition of glutathione adducts via transient formation of oxidised cysteine sulfenic acid intermediates [17] [18].

S-glutathionylation of CA3 is believed to help protect and aid recovery of cells from the damaging effects of oxidative agents. Previous studies showing that CA3 has anti-oxidant activity and that the protein is S-glutathionylated has led to speculation that the two processes are connected [18]. Direct functional evidence for this is lacking. In this study advantage is taken of our previous analysis in Rat1 fibroblast cells [15]. Derivative Evi1 proto-oncoprotein expressing Rat1 fibroblast cells, designated 5.61, have low ca3 and increased sensitivity to oxidative stress but become resistant upon restoration of ca3 levels by ectopic expression of Ca3. 5.61 cells are used here to explore the relationship between CA3 S-glutathionylation and CA3 anti-oxidant activity.

2. Materials & Methods

Cell Culture

Rat1 fibroblast cells and Evi1 expressing Rat1 cell population 5.61 cells have been described previously [15]. Rat1 and 5.61 cells were cultured in complete

medium (CM) comprising Dulbecco's Modified Eagle's Medium (Lonza Group Ltd, Basel, Switzerland, BE12-604F) supplemented with 5% newborn calf serum (Sigma-Aldrich, Poole, UK, N4637) and 2.5 mM glutamine, 50 μg/ml penicillin, 50 units/ml streptomycin (Lonza Group Ltd., BE17-605E & BE17-603E), 500 μg/ml G418 (5.61 cells only, Invitrogen, Paisley, UK), 37˚C, 5% CO_2. For hydrogen peroxide (H_2O_2) treatment, cells were incubated in CM supplemented with 750 mM H_2O_2 (Sigma-Aldrich, 21676) for 16 hrs.

Preparation of Plasmid DNA

Plasmids pCMVsport6Ca3 (I.M.A.G.E. Id 4195712), pCMVsport6Ca2 (I.M.A.G.E. Id 6479187) and pRLCMV have all been described previously (Source Bioscience, geneservice, Cambridge, UK; Stratagene, La Jolla, CA, USA). Plasmid DNA's were prepared by affinity chromatography using Nucleobond® PC500EF gravity flow columns according to manufacturer's instruction (Macherey-Nagal GmbH & Co. Kg, Düren, Germany).

Site Directed Mutagenesis

Point mutations of Ca2 and Ca3 gene sequences were created by Quick Change™ XL site directed mutagenesis using either pCMVsport6Ca2 or pCMVsport6Ca3 DNA, according to the manufacturer's instructions (Agilent technologies, USA). Briefly, 50 ng of plasmid DNA was mixed with 125 ng forward and reverse primers, 1 μl dNTP's, Pfu Turbo DNA polymerase (2.5 U) in 50 μl and incubated 95˚C, 30 sec then 14 cycles of 95˚C 30 sec, 55˚C 1 min followed by 68˚C 6 min in a programmable thermocycler (MJ Scientific, Hampton, New Hampshire, USA, PTC-100). N186C (Ca2) and C181S (Ca3) mutant plasmid DNA's were used as template for site directed mutagenesis to create double mutants S181CN186C (Ca2) and C181SC186S (Ca3) respectively. Oligonucleotide primers for site directed mutagenesis were designed using the site directed mutagenesis primer design tool (Agilent technologies) and synthesized by Integrated DNA Technologies (BVBA, Leuven, Belgium). Ca2 S181C FP: GCTAACTTTGATCCTTGCTGCCTTCTTCCTGGAAAC; Ca2 S181C RP: GTTTCCAGGAAGAAGGCAGCAAGGATCAAAGTTAGC; Ca2 N186C FP: TTGCTCCCTTCTTCCTGGATGCTTGGACTACTGGACATAT; Ca2 N186C RP: ATATGTCCAGTAGTCCAAGCATCCAGGAAGAAGGGAGCAA;

Ca2 S181CN186C FP: GATCCTTGCTGCCTTCTTCCTGGATGCTTGGACTAC; Ca2 S181CN186C RP: GTAGTCCAAGCATCCAGGAAGAAGGCAGCAAGGA

TC; Ca3 C181S FP: TTTTACACACTTTGACCCATCAAGCC

TGTTCCCTGCTTGCCG; Ca3 C181S RP: CGGCAAGCAGGGAACAGGCTTGA

TGGGTCAAAGTGTGTAAAA; Ca3 C186S FP: CATGCCTGTTCCCTGCTAGCC

GGGACT; Ca3 C186S RP: AGTCCCGGCTAGCAGGGAACAGGCATG;

Ca3 C181SC186S FP: CAAGCCTGTTCCCTGCTAGCCGGGACTATTGGACCT

ACC; Ca3 C181SC186S RP:
GGTAGGTCCAATAGTCCCGGCTAGCAGGGAACAGGCTTG.

Sequencing

Point mutation of Ca2 and Ca3 gene sequences were verified by partial sequencing of plasmid DNA (DNA Analysis Facility, Human Genetics Unit, Ninewells Hospital, Dundee, UK). DNA sequence analysis was performed using FinchTV version 1.4.0.

DNA Mediated Transfection

5.61 cells were transfected with 1 μg plasmid DNA using Fugene6® (Roche Diagnostics GmbH, Mannheim, Germany, 11815091001). 1×10^5 cells were incubated with a 1:6 ratio DNA: FuGENE6®, prepared as described by the manufacturer, for 48 hrs.

Caspase 3 Assay

Cells were incubated in 96 well dishes (Costar 3917) and apoptosis determined by the Caspase 3/7-Glo® assay according to the manufacturer's instructions (Promega, G8090), measuring luminescence with a Fluostar OPTIMA luminometer (BMG LABTECH).

Western Blot Analysis

Protein extracts, SDS polyacrylamide gel electrophoresis and western blotting were performed as described previously [15] with either *α*-ca3 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA, E-19), *α*-ca2 (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA, C-10) or *α*-gapdh (Fitzgerald Industries, North Acton, MA, USA, 6C5) and diluted 1/1000 (E-19), 1/200 (C-10) or 1/5000 (6C5). Appropriate HRP conjugated *α*-goat (Sigma-Aldrich, A5420) or *α*-mouse (Sigma-Aldrich, A9044) IgG secondary antibodies were used at 1/5000 dilutions and detection was performed by enhanced chemiluminescence (Pierce, Rockford, IL, USA, 32209).

Statistical Analysis

Statistical significance was determined by two-way ANOVA using GraphPad PRISM® 7.0c software. $P \leq 0.05$ was considered significant.

3. Results

Ca3 but not Ca2 protects cells from oxidative stress

Murine Ca2 and Ca3 proteins show 60% amino acid identity (**Figure 1**). Initially we examined and compared the anti-oxidant activity of these two proteins in cells. Evi1 transformed Rat1 cells (5.61) were chosen for this purpose as they have previously been shown to express low levels of ca3 [15] and they are −ve for ca2. Both genes were transiently expressed for 48 hrs in 5.61 cells using pCMVsport6Ca2 and pCMVsport6Ca3 expression vectors as described in materials and methods. Western blot analysis with *α*-ca3 (Santa Cruz E-19) antibodies shows elevated levels of Ca3 in pCMVsport6Ca3 transfected 5.61 cells relative to parental (UT) or empty vector (pRCCMV) transfected cells (**Figure 2(a)**). Similarly, western blot analysis with *α*-ca2 (Santa Cruz D-8) shows abundant

```
Ca3     MAKEWGYASHNGPEHWHELYPIAKGDNQSPIELHTKDIRHDPSLQPWSVS 50
Ca2     -SHH---SKS----NW-KEF---N--R---VDID-GTAQ-------LLIC 50

        YDPGSAKTILNNGKTCRVVFDDTFDRSMLRGGPLSGPYRLRQFHLHWGSS 100
        --KVAS-S-V---HSFN-E---SQ-FAV-KE-----S---I---F----- 100

        DDHGSEHTVDGVKYAAELHLVHWNPKYNTFGEALKQPDGIAVVGIFLKIG 150
        -GQ------NKK-----------T--GD--K-VQH------L------- 150

        REKGEFQILLDALDKIKTKGKEAPFNHFDPSCLFPACRDYWTYHGSFTTP 200
        PASQGL-KITE--HS------R-A-AN---CS-L-GNL-----P--L--- 200

        PCEECIVWLLLKEPMTVSSDQMAKLRSLFASAENEPPVPLVGNWRPPQPI 250
        -LL--VT--V----I----E--SHF-K-NFNS-G-AEELM-D----A--- 250

        KGRVVRASFK 260
        -N-KIK---- 260
```

Figure 1. Comparison of the primary amino acid sequence of murine Ca2 (Accession number NM_009801) and Ca3 (Accession number NM_007606) proteins. Identity of amino acid sequences are indicated using the single letter code. Regions of Ca2 identical with Ca3 are indicated by –. Cysteine residues 181 and 186, present in Ca3 but absent from Ca2 are highlighted by a boxed **C** in bold.

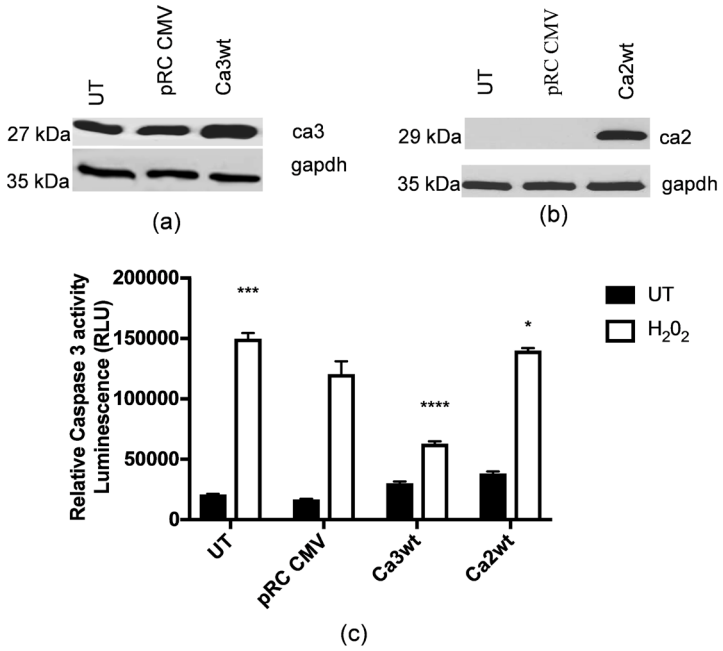

Figure 2. Caspase 3 activity in H_2O_2 treated 5.61 cells with enforced expression of Ca2 and Ca3. (a) and (b) show representative examples of western blot analysis of whole cell extracts derived from untransfected (UT) 5.61 cells or 5.61 cells transfected with empty vector (pRC CMV), Ca2 (Ca2wt) or Ca3 (Ca3wt) expression vectors for 48 hrs with *α*-ca2 (C-10), *α*-ca3 (E-19) and *α*-gapdh (6C5) antibodies. The expected 29 kDa Ca2, 27 kDa Ca3 and 35 kDa gapdh proteins are indicated; (c) shows a histogram of relative caspase 3 catalytic activity in untransfected (UT), empty vector (pRC CMV) transfected and Ca2 (Ca2wt) or Ca3 (Ca3wt) overexpressing 5.61 cells + (white columns) or – (black columns) 16 hrs H_2O_2. Histogram is the mean value of three measurements and the error bars the SEM. Statistical analysis shows two-way ANOVA and Dunnetts multiple comparisons test of H_2O_2 treated pRC CMV vs UT or Ca3wt or Ca2wt, *P < 0.05, ***P < 0.001, ****P < 0.0001.

Ca2 protein in pCMVsport6Ca2 transfected 5.61 cells which is absent from parental (UT) and empty vector (pRCCMV) transfected cells (**Figure 2(b)**). In each case, western blot analysis with *α*-gapdh (Santa Cruz 6C5) confirmed even loading of proteins.

Next, we investigated the anti-oxidant activity of Ca2 and Ca3 in 5.61 cells. Cells were transiently transfected with Ca2 or Ca3 expression vectors as described above and then treated with 750 μM H_2O_2 for 16 hrs. H_2O_2 induced apoptosis was examined by measuring caspase 3 catalytic activity as described in materials and methods. Ca2, Ca3 or empty vector control cells all show low caspase 3 catalytic activity in the absence of H_2O_2 treatment (**Figure 2(c)**). Caspase 3 catalytic activity is induced in each case following H_2O_2 treatment but is significantly less in Ca3 expressing cells (**Figure 2(c)**, pRC CMV vs Ca3wt, ****P < 0.0001). In contrast, Ca2 expression does not protect 5.61 cells from H_2O_2 induced apoptosis (**Figure 2(c)**, Ca2wt). These data confirm that Ca3 protects cells from oxidative stress induced apoptosis and that this property is not shared by the Ca2 isozyme.

Cysteines 181 and 186 are necessary for Ca3 anti-oxidant activity in Rat1 cells

Reversible oxidative stress induced ca3 S-glutathionylation occurs on cysteine residues 181 and 186 [18]. A full length wild type (wt) Ca3genecDNA in pCMVsport 6 was mutated by site directed mutagenesis (materials & methods) to encode Ca3serine at position 181 (Ca3C181S), Ca3serine at position 186 (Ca3C186S) single mutant or Ca3 serine at position 181 and 186 (Ca3C181SC186S) double mutant proteins, that have previously been shown to inhibit S-glutathionylation [18]. To confirm their expression, the wt and mutant Ca3 proteins were each transiently over-expressed by transfection of the various constructs in 5.61 cells. Western blot analysis of whole cell extracts derived from the transfected cells with *α*-ca3 shows over-expression of wt and mutant 27 KDa Ca3 proteins in transfected cells (**Figure 3(a)**) relative to the endogenous protein in empty vector transfected 5.61 cells (**Figure 3(a)**, pRCCMV). In each case, western blot analysis with *α*-gapdh confirmed even loading of proteins.

The mutant Ca3 proteins were next examined for anti-oxidant activity. 5.61 cells transiently transfected with constructs encoding either wt or mutant Ca3 proteins were examined for caspase 3 catalytic activity (materials and methods) either with or without 750 μM H_2O_2 treatment for 16 hrs. The results show low caspase 3 enzyme catalytic activity is significantly induced following exposure to H_2O_2 in each case (**Figure 3(b)**). As before, there is a highly significant reduction in caspase 3 enzyme catalytic activity in cells with enforced expression of wt Ca3 (**Figure 3(b)** pRC CMV vs Ca3wt, ****P < 0.0001) confirming anti-oxidant activity. However, the H_2O_2 induced caspase 3 enzyme catalytic activity is induced to either a greater (**Figure 3(b)** pRC CMV vs UT ****P < 0.0001 or Ca3C181S **P < 0.01 or Ca3C186S **P < 0.01) or similar extent (**Figure 3(b)** pRC CMV vs Ca3C181SC186S) in all other cells that are either untransfected (UT) or

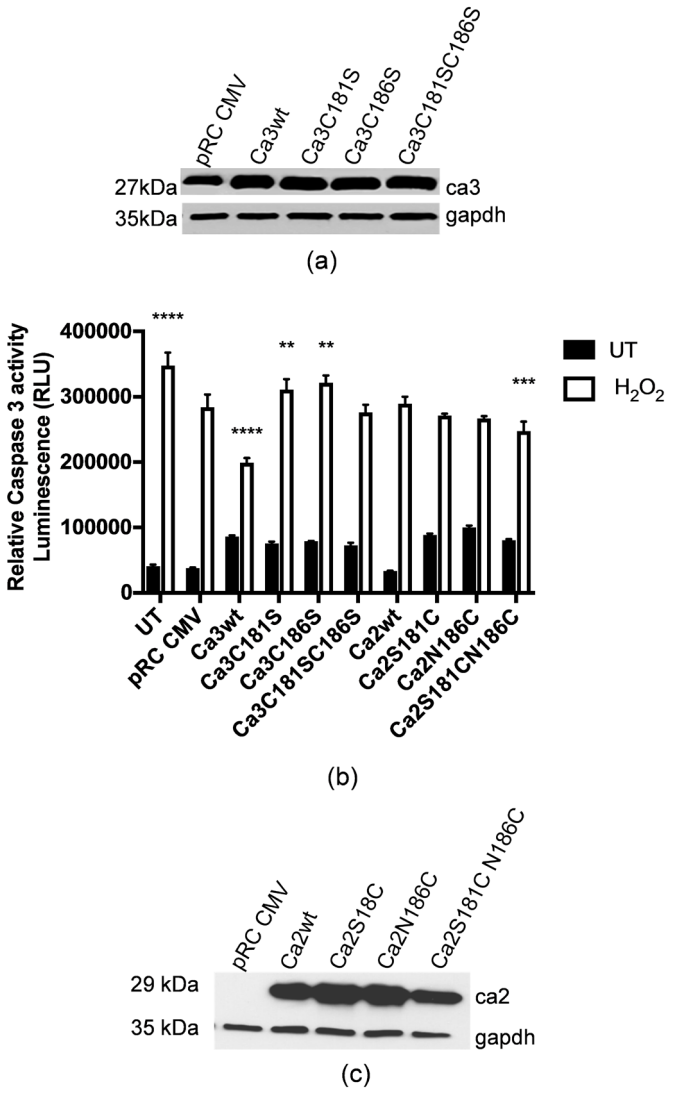

Figure 3. Caspase 3 activity in H_2O_2 treated 5.61 cells with enforced expression of Ca3 and Ca2 encoding wt, cysteine 181 and 186 mutant proteins. (a) shows western blot analysis of whole cell extracts derived from transfected 5.61cells with the empty vector control pRC CMV or over expressing the indicated Ca3wt and mutant proteins. The antibodies used and the Ca3 and gapdh proteins detected are as indicated in figure legend 2; (b) shows a histogram of relative caspase 3 catalytic activity in untransfected (UT), empty vector (pRC CMV), Ca3wt, indicated Ca3 cysteine mutant, Ca2wt and indicated Ca2 serine or asparagine mutant overexpressing 5.61 cells + (white columns) or − (black columns) 16 hrs H_2O_2. Histogram is the mean value of three measurements and the error bars the SEM. Statistical analysis shows two-way ANOVA and Dunnetts multiple comparisons test of H_2O_2 treated pRC CMV vs UT or Ca3wt or C181S or C186S or C181SC186S or Ca2wt or Ca2S181C or Ca2N186C or Ca2S181CN186C, **P < 0.01, ***P < 0.001, ****P < 0.0001.

transfected with one of the three Ca3 mutant proteins. This shows mutant Ca3proteins are no longer protective in Rat1 cells exposed to oxidative stress.

Cysteines 181 and 186 are insufficient to confer anti-oxidant activity on Ca2 in Rat1 cells

The Ca2 amino acids serine 181 (Ca2S181C) and asparagine 186 (Ca2N186C) were converted to cysteine residues, by site directed mutagenesis of pCMVsport6Ca2 expression vector DNA, to see if this would confer anti-oxidant activity on the Ca2 protein. Ca2wt, Ca2S181C, Ca2N186C single mutant and Ca2S181CN186C double mutants were each transiently expressed in 5.61 cells (materials and methods). Western blot analysis of cell extracts with *α*-ca2 shows the presence of similar amounts of the expected wt and mutant 29KDa proteins in transiently transfected cells which is absent from cells transfected with the empty vector control (**Figure 3(c)**). In each case, western blot analysis with *α*-gapdh confirmed even loading of proteins (**Figure 3(c)**). The mutant Ca2 proteins were examined for antioxidant activity in H_2O_2treated cells as before. No significant difference was observed in H_2O_2 induced caspase 3 catalytic activity when compared to empty vector control (pRC CMV) cells (**Figure 3(b)**) but there was a minor but significant reduction with the double mutant protein (**Figure 3(b)** pRC CMV vs Ca2S181CN186C ***P < 0.001).

4. Discussion

It is now widely accepted that CA3 undergoes S-glutathionylation [19] and that this post-translational modification is elevated by either chemical [16] [20], including H_2O_2 [21] or exercise [11] mediated oxidative stress in cultured cells and intact tissues in various mammalian species examined. X-ray crystallography shows that both rat ca3 and S-glutathionylated ca3 are structurally very similar and that two surface exposed cysteine residues (C181 and C186) participate in adduct formation [17]. Substitution of both C181 and C186 for serine completely abolishes S-glutathionylation of Ca3 [18]. We show in this study that Ca3 C181 and C186 are both essential for *in vivo* Ca3 anti-oxidant activity and that S-glutathionylation is therefore an essential feature of the mechanism by which this protein participates in protecting cells from oxidative stress.

Mutation of either C181, C186 or both to serine residues are equally effective at inhibition of Ca3 anti-oxidant activity. Previous studies show that C186 is preferentially S-glutathionylated relative to C181, suggesting that this residue might be more important in anti-oxidant activity [18]. However, the same studies also show that the efficiency of C186 S-glutathionylation is significantly reduced in the C181 mutant Ca3 protein (70% reduction). These observations are consistent with our results that mutation of either one or both of these cysteine residues to encode a serine has a significant impact on Ca3 biological activity as an anti-oxidant.

Our results also confirm previous studies [14] that the closely related Ca3 protein, Ca2, does not share anti-oxidant activity. Furthermore, we show that the introduction of cysteines at positions 181 and 186 of the Ca2 protein is insufficient to confer anti-oxidant activity although there is a minor increase in the presence of both residues. This result suggests other factors, which might impact on the efficiency of S-glutathionylation, are required for anti-oxidant activity.

The S-glutathionylated cysteine residues in Ca3 have a low pKa [18] and S-glutathionylation is affected positively and negatively by lysine 211 and glutamic acid 188/aspartic acid 212 respectively. The pKa of the cysteine residues in mutant Ca2 have not been determined, however the lysine, aspartic acid and glutamic acid amino acids are conserved in Ca2. The S-glutathionylation process can occur spontaneously (reviewed in [22]) but can be catalysed. Glutathione-S-Transferase π (GSTP) catalyses S-glutathionylation of both 1-CYS peroxiredoxin [23] and cardiac aldose reductase [24]. Homozygous KO mice depleted of GSTP show a general reduction in oxidative stress induced protein S-glutathionylation [25] suggesting this protein might be involved in conjugation of glutathione to other proteins, including Ca3. Therefore, there might be other molecular determinants of Ca3 S-glutathionylation besides C181 and C186 that are absent from Ca2.

The CA3 C181 and C186 residues are conserved in all mammalian species examined, including human, rat, murine and bovine, but only C181 is found in xenopus and neither residues are observed in chicken or zebrafish (data not shown). This suggests that only mammalian CA3 has evolved anti-oxidant activity, but this would need to be tested experimentally as other cysteine residues might be S-glutathionylated in non-mammalian species. For example, the CA3 isozyme CAVII is also S-glutathionylated but at cysteine residues C183 and C217 [26].

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Conflicts of Interest

The authors declare no conflict of interest.

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